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Thermophilic bacterium *Caldimonas taiwanensis* produces poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from starch and valerate as carbon sources

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ABSTRACT

Caldimonas taiwanensis accumulated polyhydroxybutyrate (PHB) at 55 °C from gluconate, fructose, maltose, and glycerol under nitrogen-limited condition. The PHB content peaked at 14 h after inoculation from gluconate. *C. taiwanensis* did not grow or accumulate PHA from fatty acids as the sole carbon source; however, it incorporated 3-hydroxyvalerate (3-HV) into PHB polymer from gluconate and valerate as a mixed carbon source. By adjusting the valerate concentration, the molar fraction of 3-HV could be modulated from 10 mol% to 95 mol%. Fatty acid valerate substantially inhibited cell growth and PHA accumulation with the addition of as little as 5 mM to the medium. Supplementing the medium with yeast extract overcame the inhibition, which enhanced not only the yield of biomass but also PHA productivity. The *in vivo* substrate specificity of PHA synthase ranged from C_4 to C_6. In addition, *C. taiwanensis* also incorporated a wide range of 3-HV into PHA from soluble starch and valerate as a mixed carbon source. Food-grade starches made from cassava, corn, potato, sweet potato and wheat respectively mixed with valerate were studied for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] production. In this study, *C. taiwanensis* exhibited high promise for reducing the production cost of P(3HB-*co*-3HV).

1. Introduction

Polyhydroxyalkanoates (PHAs) are a sort of biological polyesters, which function as carbon and energy reserves in prokaryotic cells; many different bacteria synthesize PHA when a carbon source is provided in excess and one essential growth nutrient is limited [1]. The biological polyesters have attracted much attention because of the similarities of their physical characteristics to those of petrochemical polyesters such as polypropylene. The biocompatible and biodegradable features of biopolyesters make them good candidates for biodegradable plastics. Poly(3-hydroxybutyrate-*co*-3- hydroxyvalerate) [P(3HB-*co*-3HV)] containing low molar fraction of 3-hydroxyvalerate (3-HV), the first such industrial product, was produced by ICI Ltd. in 1982 and sold under the trademark Biopol®. It is more flexible and tougher than homopolymer and was produced on an industrial scale by the fermentation of *Cupriavidus necator* from glucose and propionic acid [2]. Production of P(3HB-*co*-3HV) with high 3-HV molar fraction was studied on bacteria such as *Chromobacterium violaceum* [3], *Delftia acidovorans* [4], and *Paracoccus denitrificans* [5] because of their different physical properties and applications in various fields. The major drawback of PHA in terms of commercialization was the high production cost of PHA compared with petrochemical-based plastics [6,7].

Some reports have focused on the economic evaluations for PHA production. The entire process for PHA production, including provision of the raw materials (carbon source), strain selection, development of fermentation strategy, and recovery of PHA, have been analyzed [6,7]. Amongst the above factors, the cost of raw materials accounts for almost half of the total production cost. Hence, it is critical to lower the production cost of PHA by employing a bacterial strain capable of using a cheaper carbon source [8]. Starch or hydrolysed starch, cellulose and hemicellulose, as well as sucrose and cheese whey have all been proposed as economical carbon sources [6]. Starch is one of the most abundant and most easily renewable carbon sources. Recently, there have been several reports on producing PHB from starch or hydrolyzed starch via bacterial fermentation [8–11].

C. taiwanensis, a thermophile isolated from a hot spring in Southern Taiwan, is capable of accumulating PHB granules in the cell from glucose [12]. The intrinsic starch-digesting enzymes detected in the cultural broth indicates that *C. taiwanensis* is able to accumulate PHB from starch directly, without digestion of the starch

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prior to fermentation. In this study, we report on the ability of *C. taiwanensis* to produce high yields of P(3HB-*co*-3HV) from starch. The modulation of 3-HV composition, *in vivo* substrate specificity of PHA synthase, and production of P(3HB-*co*-3HV) from starch and valerate as a mixed carbon source are analyzed.

2. Materials and methods

2.1. Bacterial strain and growth conditions

C. taiwanensis was cultivated on three fold diluted Luria-Bertani (1/3× LB) broth (Bacto-tryptone 3.3 g l^{-1}, yeast extract 1.7 g l^{-1}, and sodium chloride 3.3 g l^{-1}) or LB agar plate at 45 °C for routine bacteria maintainance. Mineral salt (MS) medium contained 4.45 g l^{-1} of $Na_2HPO_4{\cdot}2H_2O$, 1.5 g l^{-1} of KH_2PO_4, 0.2 g l^{-1} of $MgSO_4{\cdot}7H_2O$, 1 g l^{-1} of $(NH_4)_2SO_4$, 60 mg l^{-1} of ferrous ammonium citrate, 10 mg l^{-1} of $CaCl_2{\cdot}2H_2O$, and 1 ml l^{-1} of trace element was used for the experiment of PHA accumulation [13].

2.2. Production of PHA

Cells grown on 1/3× LB medium for 12 h at 45 °C were inoculated to a 250 ml flask with 30 ml MS medium. Filter-sterilized carbon sources, except starches, were added to the medium as indicated in the text. The cells were cultured at 55 °C on an orbital shaker (200 rpm min^{-1}) for indicated time. The PHA accumulated cells were harvested, washed with nitrogen free MS medium, lyophilized, and subjected to methylation for gas chromatography (GC) analysis. For substrate specificity analysis, *C. taiwanensis* was subjected to PHA accumulation experiments using 1.5% of gluconate mixed with 0.1% of fatty acids as carbon sources. When the fatty acid was one component of the mixed carbon sources, the cultural temperature was adjusted to 50 °C and the MS medium supplemented with 0.75 g l^{-1} of yeast extract.

For PHA accumulation from valerate and starch as a mixed carbon source, 15 ml of the log phase cultured bacteria (OD_{600} at ca. 0.7) was harvested by centrifugation at 25 °C and washed once with MS medium before being inoculated to a 250 ml flask containing 30 ml MS medium. After 16 h additional cultivation at 50 °C, the cells were harvested and lyophilized for GC analysis.

The fatty acids were neutralized with sodium hydroxide into sodium salt before being used as the carbon source. All the carbon sources, including gluconate and fatty acids, were prepared as 20% solutions in water and filter-sterilized. The soluble starch and food-grade starch solution (3%) was freshly prepared in MS medium by heating to boiling and mixing with magnetic stirring for 20 min prior to PHA accumulation experiments. It was ready for use after cooling to room temperature.

2.3. Analysis of polyhydroxyalkanoate in cells

The PHA content and monomer composition were analyzed by GC. Approximately 10 mg of lyophilized cells was subjected to methanolysis in the presence of 15% sulfuric acid as previously described [14]. The resulting methyl esters of constituent 3-hydroxyalkanoic aids were assayed by GC according to Brandl et al. [15]. GC analysis was performed on an Agilent Plus equipped with a HP-5 capillary column (30 m × 0.25 mm; 0.25 μm film thickness) and a flame-ionization detector.

3. Results

3.1. The growth and PHB accumulation

C. taiwanensis was capable of growing in 1/3× LB medium but not in rich medium such as 2× YT medium at 55 °C. Furthermore, it accumulated 14% (dry w/w) of PHB in 1/3× LB medium containing 1.5% gluconate as carbon source. Higher than 50% of PHB was obtainable under nitrogen limitation condition. It accumulated 70%, 62%, 60%, and 52% of PHB in the cells at 55 °C (optimal growth temperature) from sodium gluconate, fructose, maltose and glycerol as sole carbon source, respectively, under nitrogen-limited condition (C/N molar ratio = 30) but not from lactose or sucrose (data not shown). The effect of nitrogen limitation on PHB accumulation was analyzed by performing PHB accumulation on various molar ratio of carbon to nitrogen (C/N molar ratio) condition. The results suggested that the optimal C/N molar ratio for PHB accumulation was 30; however, the cell still accumulated 45% (dry w/w) of PHB from gluconate when the C/N molar ratio was five (data not shown). To determine the cultivation time for PHB accumulation in flask experiment, the growth and PHB content of *C. taiwanensis* were monitored at 55 °C with optical density measurement (OD_{600}) and GC analysis, respectively, in which 1.5% gluconate was provided as the carbon source. The results revealed that the PHB content simultaneously increased with the cell growth and reached the highest content (71 dry wt%) at 14 h (early stationary phase) after inoculation. After peaking, the PHB content gradually decreased with time (Fig. 1).

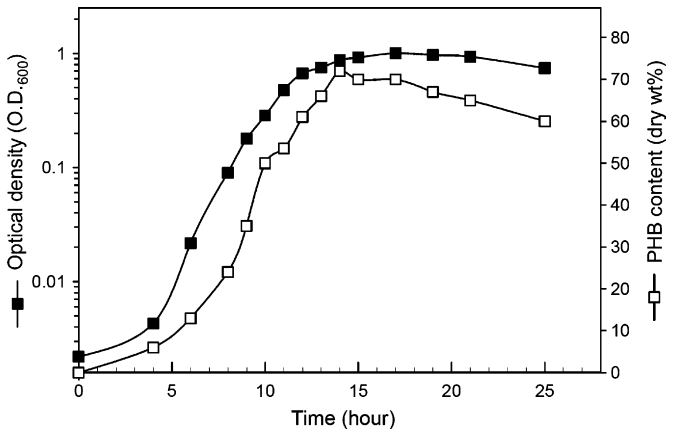

Fig. 1. The growth curve and PHA accumulation of *C. taiwanensis* at 55 °C in MS medium containing 1.5% sodium gluconate as carbon source.

3.2. Biosynthesis of P(3HB-co-3HV)

Using fatty acid as a sole carbon source, *C. taiwanensis* did not grow at 45 °C or 55 °C, and no PHA was detected, when the bacteria was grown in rich medium and then transferred to MS medium for PHA accumulation (fatty acids tested included propionate, valerate, hexanoate, heptanoate, octanoate, nonanoate, and decanoate, at concentrations ranging from 0.05% to 0.5% in MS medium). Obviously, *C. taiwanesnis* could not use fatty acid as the sole carbon source for growth or PHA accumulation. Nonetheless, it incorporated 3-HV into polymer when gluconate and valerate were provided as a mixed carbon source. Through regulation of the concentration of valerate (5 mM to 80 mM) in a gluconate contained medium, a wide molar fraction of 3-HV (10 mol% to 95 mol%) was incorporated into PHB polymer (Fig. 2). However, a low yield of biomass (0.8 dry wt g l^{-1}) was obtained when even as little as 5 mM of valerate was added to the gluconate containing medium, as compared with only gluconate as the carbon source (3.4 g l^{-1}). A high concentration of valerate (higher than 20 mM) substantially inhibited the bacterial growth and PHA accumulation (Fig. 2). The results revealed that the presence of valerate markedly inhibited

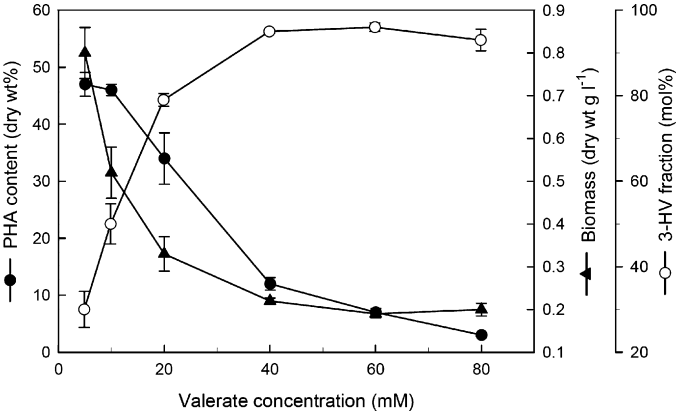

Fig. 2. The modulation of 3-HV composition in PHA accumulated by *C. taiwanensis* from various concentrations of valerate mixed with 1.5% gluconate as carbon sources. The bacteria were grown at 50 °C for 29 h.

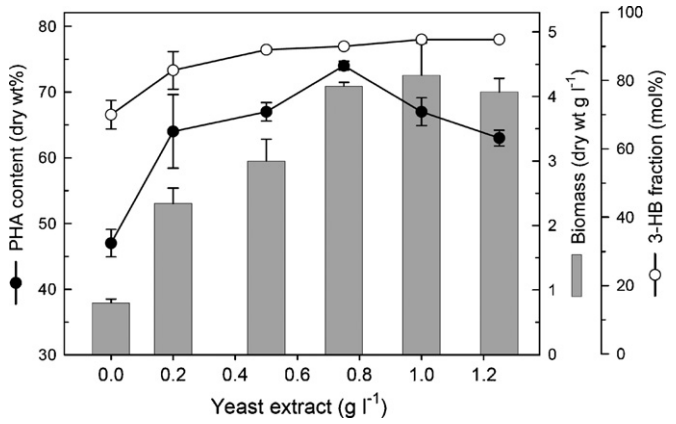

Fig. 3. The effect of yeast extract on the yield of biomass and PHA production from 1.5% gluconate and 0.05% valerate as mixed carbon source.

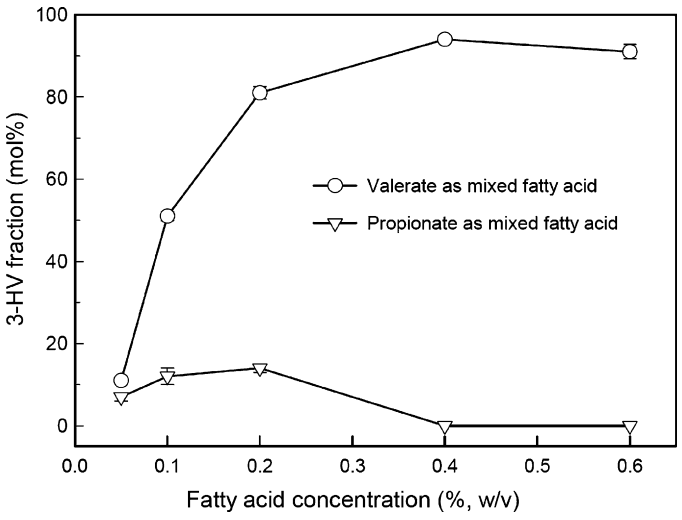

Fig. 4. The effects of valerate and propionate on the modulation of 3-HV in PHA. The 1.5% gluconate mixed with various concentrations of fatty acids were used as carbon sources.

not only the growth of the bacterium but also the PHA biosynthesis. Although the molar fraction of 3-HV could easily be modulated in this study, the low biomass and low PHA content would hamper the application to PHA production.

Supplementing the MS medium with yeast extract enhanced both the biomass yield and PHA productivity without drastically affecting the monomer compositions (Fig. 3). The effect of yeast extraction on PHA accumulation was investigated with 1.5% gluconate and 0.05% valerate as a mixed carbon source. For additions of 0.2–0.75 g l^{-1} of yeast extract, the PHA content increased with the increase in yeast extract and peaked at 0.75 g l^{-1}; further increase resulted only in decreases (Fig. 3). The trend of the amount of biomass was similar to that of PHA content, but no obvious decrease was observed at concentrations higher than 0.75 g l^{-1}. The optimal concentration of yeast extract for PHA productivity (PHA content% × biomass g l^{-1}) was thus concluded to be 0.75 g l^{-1}. In this condition, *C. taiwanensis* converted 1 g of valerate into 0.67 g of 3-HV (Fig. 3). The above results indicate that although valerate inhibited the growth of *C. taiwanensis*, it could be efficiently metabolized into 3-HV precursor for copolymerization.

Valerate and propionate are fatty acids that are commonly used for introducing 3-HV monomer into polymer [13]. Fig. 4 demonstrates the effects of valerate and propionate upon P(3HB-*co*-3HV) biosynthesis by *C. taiwanensis*. In this experiment, the medium was supplemented with 0.75 g l^{-1} of yeast extract. With valerate as the mixed fatty acid, *C. taiwanensis* incorporated 10–95 mol% of 3-HV into PHB polymer; however, only 14 mol% of 3-HV was incorporated when propionate was the mixed fatty acid (Fig. 4). In addition, higher than 0.2% of propionate almost completely inhibited the PHA accumulation and bacterial growth. Significantly, *C. taiwanensis* was capable of efficiently converting valerate into copolymer but not propionate. With valerate as the mixed carbon source, the 3-HV composition of PHA produced by *C. taiwanensis* could be widely modulated.

3.3. The in vivo substrate specificity of PHA synthase

The *in vivo* substrate specificity of PHA synthase was evaluated by analyzing the monomer composition of PHA accumulated from gluconate mixed with various fatty acids (carbon chain-lengths from 3 to 10). The monomer composition of PHA was analyzed by GC. *C. taiwanensis* was able to grow and accumulate PHA from 1.5% gluconate mixed with 0.1% of valerate, hexanoate, heptanoate, or octanoate, respectively, as carbon sources and exhibited no growth from 1.5% gluconate mixed with 0.1% nonanoate or 0.1% decanoate (Table 1). GC analysis revealed that *C. taiwanensis* was capable of incorporating trace amounts (0.5 mol%) of 3-hydroxyhexanoate (3-HHx) into polyester from hexanoate and gluconate as a mixed carbon source. The molar fraction of 3-HHx was slightly increased to 1.5 mol% when acrylic acid (2 mM), a β-oxidation inhibitor, was present in the medium (Table 1). When heptanoate was the mixed fatty acid, the molar fraction of 3-HV was 35 mol%. This was drastically enhanced up to 85 mol% when acrylic acid was added to the medium; under this condition, no 3-hydroxyheptanoate (3-HHp) was detected in the polymer (Table 1). We also tried adding 3 mM of acrylic acid to the heptanoate contained medium for PHA accumulation. However, there was still no 3-HHp detected (data not shown). The results suggested that the *in vivo* substrate specificity of PHA synthase was ranged from C_4 to C_6 and that the β-oxidation pathway seems to be the source of

Table 1
The PHA accumulation by thermophile *C. taiwanenesis* from gluconate mixed with various fatty acids as carbon sources, with the medium supplemented with 0.75 g l^{-1} yeast extract.

Carbon source	Biomass (dry wt, g l^{-1})	PHA content[a] (%, w/w)	Polymer composition (mol%)			
			3-HB	3-HV	3-HHx	3-HHp
0.1% Propionate + 1.5% Glc	2.0 ± 0.20	52 ± 4.3	88 ± 2.0	12 ± 2.0	–	–
0.1% Valerate + 1.5% Glc	1.0 ± 0.03	51 ± 2.0	49 ± 1.1	51 ± 1.1	–	–
0.1% Hexanoate + 1.5% Glc	2.7 ± 0.50	62 ± 6.5	99.5 ± 0.15	–	0.5 ± 0.15	–
0.1% Hexanoate + 1.5% Glc + 2 mM acrylic acid	1.18 ± 0.03	47 ± 6.5	98.5 ± 0.5	–	1.5 ± 0.5	
0.1% Heptanoate + 1.5% Glc	1.7 ± 0.20	33 ± 3.0	65 ± 2.6	35 ± 2.6	–	–
0.1% Heptanoate + 1.5% Glc + 2 mM acrylic acid	0.28 ± 0.03	17 ± 1.2	15 ± 0.9	85 ± 0.9	–	–
0.1% Octanoate + 1.5% Glc	0.4 ± 0.07	13 ± 3.4	100	–	–	–
0.1% Nonanoate + 1.5% Glc	No Growth	–	–	–	–	–
0.1% Decanoate + 1.5% Glc	No Growth	–	–	–	–	–

[a] The bacteria are cultivated at 50 °C for 29 h and all the data are the mean value of three individual experiments.

Table 2
The PHA accumulation by *C. taiwanensis* from commercially available starches and valerate as mixed carbon sources. Yeast extract 0.1 g l^{-1} was added in all experiments.

Carbon sources	Biomass (dry wt, g l^{-1})	PHA content[a] (%, wt/wt)	Monomer composition (mol%)	
			3-HB	3-HV
1.5% cassava starch + 0.05% valerate	2.8 ± 0.07	67 ± 2.8	87 ± 1.0	13 ± 1.0
1.5% corn starch + 0.05% valerate	3.3 ± 0.07	65 ± 3.0	90 ± 0.0	10 ± 0.0
1.5% potato + 0.05% valerate	2.6 ± 0.16	55 ± 2.6	90 ± 1.0	10 ± 1.0
1.5% sweet potato + 0.05% valerate	1.6 ± 0.07	52 ± 3.8	90 ± 2.5	10 ± 2.5
1.5% wheat starch (low gluten) + 0.05% valerate	4.1 ± 0.20	42 ± 4.0	90 ± 1.0	10 ± 1.0

[a] The bacteria is cultivated at 50 °C for 32 h.

the conversion of fatty acid into 3-hydroxyacyl-CoA for PHA copolymerization.

3.4. Biosythesis of PHB and P(3HB-co-3HV) from starches

C. taiwanensis secretes starch-degrading enzymes into the medium, which efficiently digests soluble starch into usable reducing sugars [16,17]. Here, starch was regarded as a cost-effective carbon source for PHB production. The flask experiment proved that *C. taiwanensis* could grow well and efficiently biosynthesize PHB polymer from a soluble starch (potato) (Fig. 5). It also produced P(3HB-*co*-3HV) copolymer from soluble starch and valerate as a mixed carbon source. However, the growth of *C. taiwanensis* was also significantly inhibited when valerate appeared in the soluble starch containing medium (data not shown). This phenomenon was the same as gluconate–valerate as a mixed carbon source. The effect of yeast extract on PHB accumulation and bacterial growth from soluble starch was also investigated. With soluble starch used as the sole carbon source, the yield of biomass was slightly decreased with the increase of yeast extract (Fig. 5). However, the trend in PHB content was reversed, with PHB content peaking (71%) when 0.1 g l^{-1} of yeast extract was added to the medium, after which it decreased with further increases in concentration of yeast extract (Fig. 5). The results suggested that higher than 0.1 g l^{-1} of yeast extract would slightly hamper the PHB accumulation from soluble starch, but that the presence of yeast extract would help the growth of bacteria in a medium containing soluble starch and valerate.

In further work on regulating the 3-HV fraction in PHA from soluble starch and valerate as a mixed carbon source, 0.1 g l^{-1} of yeast extract was added to all experiments. When soluble starch was mixed with various concentration of valerate, the composition of 3-HV was adjustable; however, the yields of biomass and PHA content were reduced drastically at valerate concentrations higher than 10 mM (0.1%) (Fig. 6). It is clear that higher concentrations of yeast extract are required to overcome the inhibition of growth and PHA accumulation caused by valerate, but higher than 0.1 g l^{-1} of yeast extract causes a reduction in the PHB content (Fig. 5). However, the 3-HV monomer could be changed greatly, from 7% to 95 mol%, by adjusting the valerate concentration in medium from 5 mM to 40 mM (Fig. 6). The results were similar to those found with gluconate–valerate used as the mixed carbon source (Fig. 2). Furthermore, five commercially available starches, made from cassava, corn, potato, sweet potato and wheat, were applied to PHA production. *C. taiwanensis* produced not only PHB from the above five starches (data not shown), but also P(3HB-*co*-HV) from valerate mixed with the above starches as the carbon source (Table 2). The yields of PHA were similar to those when from gluconate–valerate was used as the carbon source (Table 2 and Fig. 4). Furthermore, of the starches used in the flask experiments, *C. taiwanensis* produced the highest PHA productivity (2.15 g PHA l^{-1}, PHA content% × biomass g l^{-1}) from 1.5% corn starch mixed with 0.05% valerate as the carbon source (Table 2).

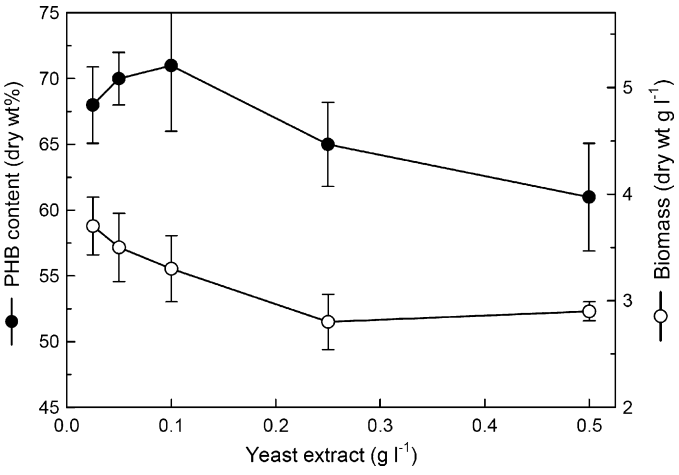

Fig. 5. The effect of yeast extract on the PHB accumulation from 1.5% soluble starch. The PHB accumulation was achieved at 50 °C for 32 h.

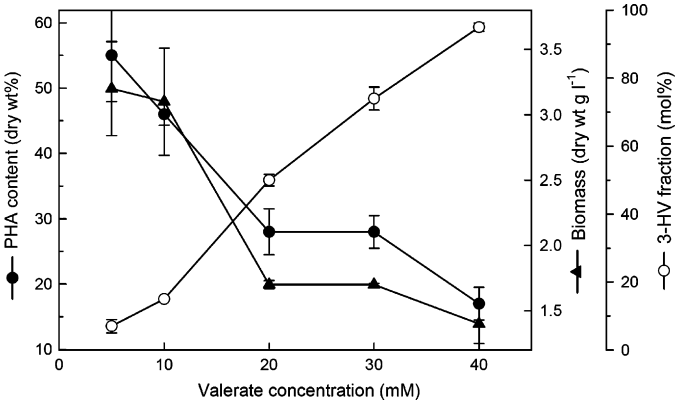

Fig. 6. The modulation of 3-HV molar fraction in PHA accumulated from 1.5% soluble starch mixed with various concentration of valerate as carbon sources.

4. Discussion

To date, few reports have focused on studying PHA accumulation by thermophile except for one thermophilic cyanobacterium, *Synechococcus* sp. [18]. Biotechnological processes running at high temperature have some advantages. For example, performing the PHB fermentation process at high temperature reduces the risk of contamination. In addition, thermophiles grow faster than mesophiles and need less time to achieve the PHA accumulation. For example, in flask experiment, mesophile *C. necator* H16 needed 48–72 h at 30 °C to reach the highest PHB accumulation from gluconate [13]. Thermophile *C. taiwanensis* needed just 14 h to achieve highest PHB accumulation at 55 °C after seeding from the same carbon source (Fig. 1).

A high concentration of fatty acid added to the medium will result in toxicity for bacterial cells and lead to the deaths of the cells [13]. *C. taiwanensis* exhibited no growth when fatty acid

was the sole carbon source (data not shown). From this result, it is clear that *C. taiwanensis* cannot use fatty acid as an energy source for growth. However, with gluconate mixed with valerate as the carbon source, the cell growth was partially recovered and 3-HV was detected in the accumulated PHA polymer. Here, gluconate worked as the carbon source for growth and PHB accumulation. The fatty acid, valerate, was mainly metabolized into PHA precursor for copolymerization but not into energy for growth. PHA accumulation performed at 55 °C will obtain a low yield of biomass and low PHA content when valerate is one component of the mixed carbon source (data not shown). The yields of biomass and PHA content recovered when the cultivation temperature decreased to 50 °C. This phenomenon may be explained by the fact that the higher temperature results in higher solubility of the fatty acid and the growth inhibition caused by the fatty acid [19].

Although fatty acid markedly inhibited the growth of *C. taiwanensis*, PHA accumulation from gluconate mixed with fatty acids was performed with the help of yeast extract. In Fig. 3, the percentage of 3-HB was slightly raised with the increase in yeast extract concentration. The result could be deduced from the limited valerate (0.05%) in the medium. Once the biomass and PHA yield were greatly enhanced by supplementing the medium with yeast extract, the percentage of 3-HV would be slightly decreased because limited valerate (0.05%) was available comparing with gluconate (1.5%) for P(3HB-*co*-3HV) biosynthesis. The monomer composition of accumulated PHA exhibited the range of *in vivo* substrate specificity of PHA synthase. In this experiment, *C. taiwanensis* incorporated trace amount of 3-HHx monomer into polymer from gluconate and hexanoate as the mixed carbon source. Furthermore, a slight increase of 3-HHx was observed after the addition of acrylic acid, a β-oxidation pathway inhibitor [20], to the medium. This suggested that the β-oxidation pathway could be the pathway for converting fatty acids into 3-hydroxyacyl-CoA for PHA biosynthesis. The metabolic pathway of fatty acids for PHA biosynthesis is similar to that of the mesophile PHA producer *C. necator* H16 [21]. Here, 3-HHx was suggested as a poor substrate for the PHA synthase. The PHA accumulation from heptanoate and gluconate as the mixed carbon source, the composition of 3-HV of polymer increased drastically but no 3-HHp detected when acrylic acid (2 mM) was added to the medium (Table 1). Furthermore, there was still no 3-HHp detected even higher concentration of acrylic acid (3 mM) added to the medium (data not shown). This evidence strongly suggested that the PHA synthase was capable of efficiently copolymerizing 3-HB and 3-HV but not 3-HHx. And 3-HHp could not be recognized by the PHA synthase.

C. taiwanensis incorporated a wide range of 3-HV (10–95 mol%) into polymer from valerate, but not propionate (Fig. 4). Furthermore, higher than a 0.2% of propionate significantly inhibited the growth of bacteria, and no PHA was detected in the cell. Based on the results of Slater et al. [22], *C. necator* H16 possesses β-ketothiolases, BktB and BktC, which efficiently condensed propionyl-CoA and acetyl-CoA into β-hydroxyvaleryl-CoA. It supports *C. necator* H16 is capable of efficiently metabolizing propionic acid into P(3HB-*co*-3HV). Accordingly, it may infer that *C. taiwanensis* does not possess such function of β-ketothiolase to support an efficient metabolic pathway for P(3HB-*co*-3HV) biosynthesis from propionic acid. Unlike previous reports, such as *C. violaceum* synthesizing PHV homopolymer from valerate [3], *D. acidovorans* synthesizing 90 mol% of 3-HV from valerate [4], and *P. denitrificans* synthesizing 3-HV homopolymer from *n*-pentanol [5], *C. taiwanensis* synthesized P(3HB-*co*-3HV) with 95 mol% of 3-HV fraction from soluble starch or gluconate mixed with valerate as the mixed carbon source (Figs. 2 and 6). More importantly, the ability of *C. taiwanensis* to efficiently convert valerate into 3-HV is crucial for reducing the cost of P(3HB-*co*-3HV) production, because the production cost of P(3HB-*co*-3HV) increases linearly with the increase of 3-HV fraction in P(3HB-*co*-3HV), and valerate is relatively expensive [7].

The bacteria barely grew in the starch and valerate contained medium when inoculated volume of culture was low. The problem was overcome using high volume of inoculation (described in materials and methods). When overnight cultured bacteria were the seed culture for PHA accumulation from starch and valerate, a low PHA content was detected in the cell (data not shown). However, log phase cultured bacteria (OD_{600} at ca. 0.7) were the seed culture, high PHA accumulation was observed. Nonetheless, *C. taiwanensis* biosynthesized PHB from soluble starch regardless of whether overnight cultured bacteria or log phase bacteria were the seed culture. Accordingly, it is clear that fast growth of cells was helpful for overcoming the toxicity caused by valerate.

Starch is one of the most abundant and cheap carbon sources in the world. The major resources for starch production and consumption worldwide are rice, wheat, corn, and potato. Generally, starch was previously hydrolyzed into glucose or oligosaccharides by a two-step process, liquefaction and saccharification, prior to fermentation. The use of commercial enzymes entails an extra expense, thus increasing the production cost of PHB fermentation. *C. taiwanensis* possesses intrinsic starch-degrading enzymes, which can directly hydrolyze starch into a bacteria-usable carbon source for PHB production during fermentation. In this study, five major resources of starch production and consumption, rice, wheat, corn, and potatoes were respectively mixed with valerate as the carbon source for PHA accumulation experiments. *C. taiwanensis* biosynthesized PHA directly from starches, but the yield of PHA and biomass were varied form starch to starch (Table 2). The results may derive from various compositions of amylose and amylopectin between starches. The ability of degrading amylose and amylopectin of the enzyme would be the key factor, which reflects the amount of usable saccharides in the medium for PHA biosynthesis. Wheat starch yielded the highest biomass, but the lowest PHA content among the used starches (Table 2). We inferred that the wheat starch contains protein (gluten), which provided nitrogen source for bacterial growth and the higher yield of biomass. Contrary, higher content of nitrogen in the medium would slightly lower the PHA accumulation. The results showed that *C. taiwanensis* produced not only PHB from the starches, but also P(3HB-*co*-3HV) from a mixed carbon source comprised of starches and valerate. This characteristic is likely to be useful in lowering the PHA production cost.

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References

[1] Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 1990;54:450–72.
[2] Byrom D. Polymer synthesis by microorganisms: technology and economics. Trends Biotechnol 1987;5:246–50.
[3] Steinbüchel A, Debzi E, Marchessault TH, Timm A. Synthesis and production of poly(3-hydroxyvaleric acid) homopolyester by *Chromobacterium violaceum*. Appl Microbiol Biotechnol 1993;39:443–9.
[4] Loo CY, Sudesh K. Biosynthesis and native granule characteristics of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in *Delftia acidovorans*. Int J Biol Macromol 2007;40:466–71.
[5] Yamane T, Chen XF, Ueda S. Growth-associated production of poly(3-hydroxyvalerate) from *n*-pentanol by a methylotrophic bacterium, *Paracoccus denitrificans*. Appl Environ Microbiol 1996;62:380–4.
[6] Choi J, Lee SY. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol 1999;51:13–21.
[7] Choi J, Lee SY. Economic considerations in the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by bacterial fermentation. Appl Microbiol Biotechnol 2000;53:646–9.

[8] Kim BS. Production of poly(3-hyddroxybutyrate) from inexpensive substrates. Enzyme Microb Technol 2000;27:774–7.
[9] Haas R, Jin B, Zepf FT. Production of poly(3-hydroxybutyrate) from waste potato starch. Biosci Biotechnol Biochem 2008;72:253–6.
[10] Huang TY, Duan KJ, Juang SY. Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferax mediterranei*. J Ind Microbiol Biotechnol 2006;33:701–6.
[11] Quillaguamán J, Hashim S, Bento F, Mattiasson B, Hatti-Kaul R. Poly(β-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. J Appl Microbiol 2005;99:151–7.
[12] Chen WM, Chang JS, Chiu CH, Chang SC, Chen WC, Jiang CM. *Caldimonas taiwanensis* sp. nov., a amylase producing bacterium isolated from a hot spring. Syst Appl Microbiol 2005;28:415–20.
[13] Ramsay BA, Lomaliza K, Chavarie C, Dubé B, Bataille P, Ramsay JA. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl Environ Microbiol 1990;56:2093–8.
[14] Sheu DS, Lee CY. Altering the substrate specificity of polyhydroxyalkanoate synthase 1 derived from *Pseudomonas putida* GPo1 by localized semi-random mutagenesis. J Bacteriol 2004;186:4177–84.
[15] Brandl H, Gross RA, Lenz RW, Fuller RC. *Pseudomonas oleovorans* as source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 1988;54:1977–82.
[16] Chen SD, Sheu DS, Chen WM, Lo YC, Huang TI, Lin CY, et al. Dark hydrogen fermentation from hydrolyzed starch treated with recombinant amylase originating from *Caldimonas taiwanensis* On1. Biotechnol Prog 2007;23:1312–20.
[17] Chen SD, Lee KS, Lo YC, Chen WM, Wu JF, Lin CY, et al. Batch and continuous biohydrogen production from starch hydrolysate by *Clostridium* species. Int J Hydrogen Energy 2008;33:1803–12.
[18] Hai T, Hein S, Steinbüchel A. Multiple evidence for widespread and general occurrence of type-III PHA synthases in cyanobacteria and molecular characterization of the PHA synthases from two thermophilic cyanobacteria: *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. strain MA19. Microbiology 2001;147:3047–60.
[19] Koller M, Bona R, Chiellini E, Fernandes EG, Horvat P, Kutschera C, et al. Polyhydroxyalkanoate production from whey by Pseudomonas hydrogenovora. Biores Technol 2008;99:4854–63.
[20] Qi Q, Steinbüchel A, Rehm BH. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid beta-oxidation by acrylic acid. FEMS Microbiol Lett 1998;167:89–94.
[21] Green PR, Kemper J, Schechtman L, Guo L, Satkowski M, Fiedler S, et al. Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid β-oxidation inhibited *Ralstonia eutropha*. Biomacromolecules 2002;3:208–13.
[22] Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgette SR, et al. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J Bacteriol 1998;180:1979–87.